REGENERATION IN ISOLATED AND FUSED PIECES OF CLAVA LEPTOSTYLA[1]

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Modern experimental studies on regeneration in hydroids, as Peebles (1931) anticipated, have been aimed at elucidating the factors responsible for this dramatic morphogenetic response. It is well known (reviews of Child, 1929, and Barth, 1940a) that coelenterate hydranths exhibit axial gradients of metabolic activity and that distal levels exert dominance over proximal levels. Barth (1938b) demonstrated that in Tubularia circulation within the gastrovascular cavity is responsible for the maintenance of dominance, for if an isolated stem was ligatured hydranths might regenerate at both ends. The same result was attained if circulation in the coelenteron was blocked with an oil droplet (Barth, 1938b), with a bubble of oxygen or nitrogen or by means of an inserted glass rod (Rose and Rose, 1941). A series of recent papers have considered certain extrinsic factors affecting regeneration. Temperature (Moog, 1941) and the level of oxygen supply (Barth, 1938a, 1940b, 1944; Goldin, 1942b; Miller, 1937; Rose and Rose, 1941) have been shown to be important in Tubularia. Accumulation of metabolic wastes (Rose and Rose, 1941; Miller, 1942) or lowered pH (Goldin, 1942a, 1942b) prevent regeneration. Zwilling (1939) showed that hydranths could be elicited along the side of a stem of Tubularia by cutting a window through the perisarc, thereby exposing the coenosarc directly to sea water, and Goldin and Barth (1941) have investigated the reorganization of coenosarcal fragments free of perisarc. As background for further work on the physiology of regeneration, the present paper deals with the potentialities for regeneration revealed in isolated or fused pieces of hydranths of *Clava leptostyla* Agassiz. Papers by Hargitt (1906 and 1911) contain valuable descriptive information on this species and a more recent paper by Brien (1943) reports experiments on regeneration in a related species, *Clava squamata*.

Materials and Methods

During the summer of 1950 *Clava leptostyla* was found to be abundant on the Fucus attached to rocks in the intertidal zone at Salisbury Cove, Maine. Clava grows in the form of colonies of separate light orange hydranths (Fig. 1) attached to a mat of hydrorhiza firmly adherent to the substratum. Mature polyps, which are about 1 cm. in length when expanded, consist of a contractile stalk, a gonosome, tentacle-bearing region, and hypostome. The gonosome is a region possessing several short, branched gonophores bearing clusters of spherical sporosacs, which are either male or female. There is no free medusoid generation; instead a planula

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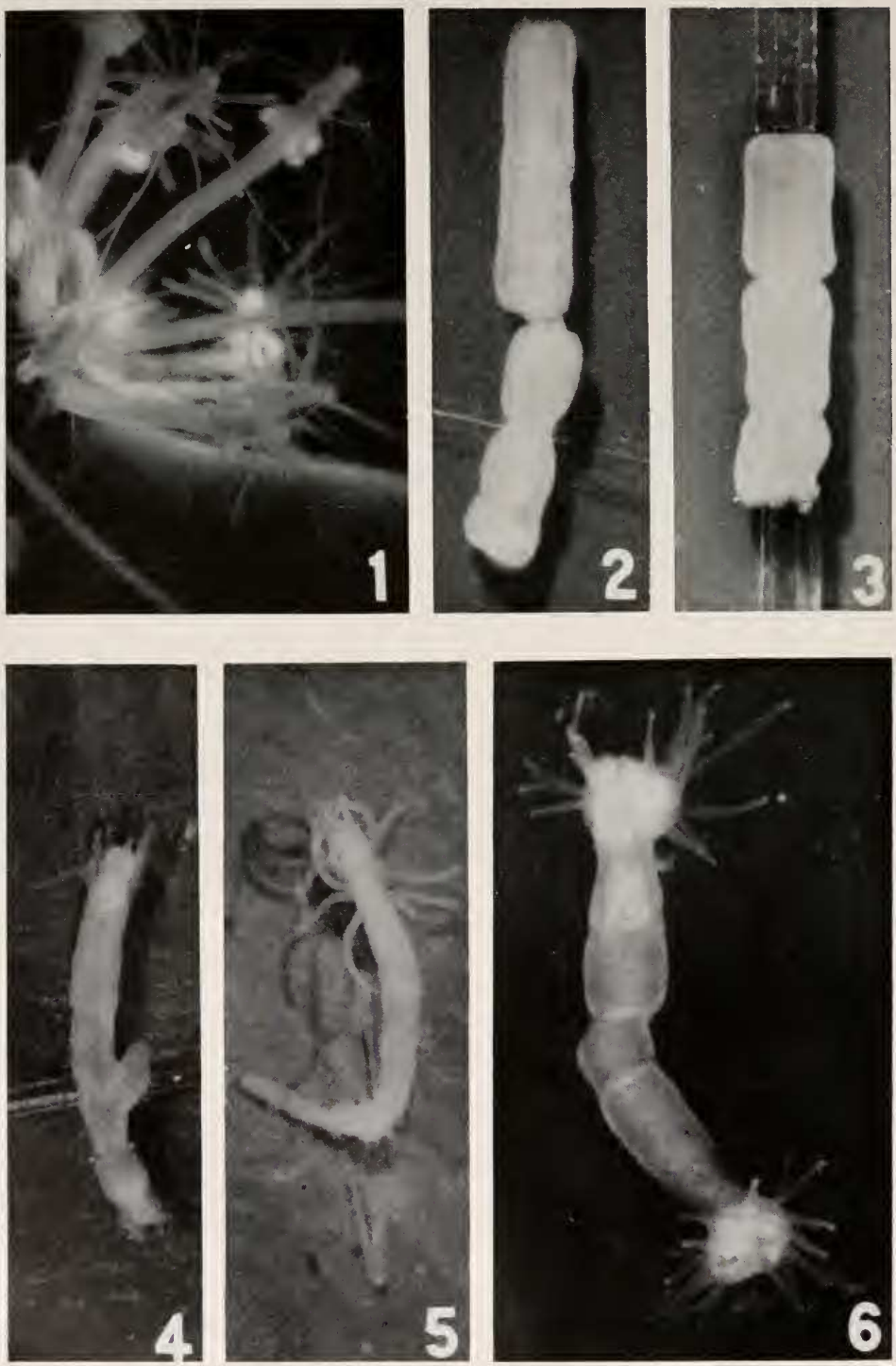
PLATE I



larva is released. There is no separate perisarc around the stalk of the hydranth but the perisarc is represented by a thin, non-living surface layer closely adherent to the ectoderm. Pieces to be isolated or fused were cut from individual hydranths with iridectomy scissors. Operations were performed in sea water and pieces were cultured at room temperature in small petri dishes immersed in finger bowls containing approximately one inch of filtered sea water. In early experiments it was noticed that isolated portions of the stalk usually rounded up and failed to undergo regeneration unless one end became attached to the culture dish. Accordingly, it became routine procedure to secure one end of an isolated piece by pinning it with a fine glass rod thrust through the tissue (Fig. 2) and into a substratum of 2% agar. Pieces to be fused were strung on a fine glass rod, then held tightly together for about 12 hours by means of glass tubing slipped over the ends of the rod and held in position with flat sections of cover glass resting on the exposed outer ends of the rod (Fig. 3). Photographs were taken with a Kodak Bantam camera mounted above one ocular of a stereoscopic microscope.

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Experiments

A. *Pieces isolated without agar substratum*

The first experiments tried were simple tests of the regenerative behavior of (1) the isolated hypostome and tentacle-bearing region, (2) the gonosome, (3) isolated stalks. Ten days after isolation 7 of the first group had become attached to the glass culture dish and were putting out hydrorhizal protuberances. Twenty-four others which remained unattached showed no basal outgrowths. It was noted by the twentieth day that most of the unattached specimens had undergone dedifferentiation of the tentacles originally present and had not regenerated. Those attached still showed their distal tentacles as well as a regenerated stalk and hydrorhiza. Isolated gonosomes (group 2) showed a marked tendency to regenerate new tentacles and hypostome at the apical end. After 10 days this was true in 8 of 23 specimens examined and one of these had regenerated two new oral ends. None

Figure 1. View of colony of *Clava leptostyla* showing expanded hydranths; gonosome with spherical sporosacs just proximal to tentacle-bearing region.

Figure 2. Specimen illustrating method of pinning with a fine glass rod used to anchor stems to substratum of 2% agar. Here rod is through middle of one member of a fused pair of stalks.

Figure 3. Specimen illustrating method of fusing pieces. Two stalks strung on a single glass rod are being fused end-to-end through pressure exerted by glass tubes slipped over each end of the rod.

Figure 4. An unusual example showing regeneration of apical structures at both ends of a stalk isolated for 15 days. Hydrorhizal outgrowth is seen at level of glass rod through original basal end of stalk; basal hydranth possibly a result of secondary regeneration.

Figure 5. A typical example of primary regeneration showing tentacles at original apical end and hydrorhizum at original basal end of stalk isolated for 15 days; pinned through basal end.

Figure 6. Specimen showing stalk of one hydranth fused between basal ends of stalks of two other complete hydranths. After 19 days no regeneration had occurred at lines of fusion.

of the gonosomal pieces became attached, hence did not develop hydrorhiza. In the third group (isolation of the stalk) many of the specimens developed bulbular enlargements with asymmetrical protuberances at one end, presumably the primordia of hydrorhiza from the original aboral end. Fifteen specimens, all still unattached, had these outgrowths after 10 days, but only 7 of the 15 had regenerated new tentacles. By the twentieth day 4 had become attached. Two of these had regenerated oral structures at the apical end of the stalk and one had a new hydranth growing out from the basal hydrorhiza.

In another group of experiments the regenerative behavior of isolated individuals of Clava in various dilutions of sea water was tested. Dilutions differing by 10% intervals from 0 to 100% sea water were tried. Forty per cent sea water or less proved too hypotonic, but the organisms showed remarkable tolerance in surviving all dilutions down to 50% sea water. Two groups of complete hydranths, isolated in 50% and 80% sea water, respectively, survived during 17 days of observation. It appeared that during this time dedifferentiation of tentacles and gonosome was more rapid than in controls kept in 100% sea water. After dedifferentiation had occurred, regeneration of new hydranths took place. One specimen in 80% sea water had regenerated 8 new hydranths at the end of the 17 days.

The above results and others not reported here indicated that two types of regenerative response should be distinguished: (1) primary regeneration resulting in the differentiation of hypostome and tentacles at the apical end, usually within 3–7 days after isolating pieces of the hydranth; and (2) secondary regeneration, meaning the delayed differentiation of completely new hydranths either from hydrorhizal outgrowths or from dedifferentiated tissue of any part of an old hydranth. The former response undoubtedly depends on the apico-basal gradient already established, while the latter represents establishment of a new center of organization in an undifferentiated primordium. The results indicated also that the mechanical factor of attachment favors both primary and secondary regeneration.

B. *Isolated stalks pinned to substratum of agar*

After the importance of attachment was realized, a series of experiments on isolated stalks was performed. Isolated pieces were pinned with a fine glass rod through one end or through the middle. One end of the rod was then thrust vertically into a substratum of 2% agar in the culture dish. This procedure favored primary regeneration (Figs. 4, 5) since pieces anchored at one end could elongate. They were thus saved from rounding up and degenerating, the usual fate of unanchored stems. The results are recorded in Table I.

Table I

Primary regeneration of isolated entire stalks pinned to substratum

Position of pin	No. of animals	Age after isolation	Number regenerating	% regenerating
Apical end	10	7 days	1	10
Middle	19	7 days	12	63
Basal end	15	7 days	11	73

It is seen that there is a distinct difference between specimens pinned through the basal end or middle of entire stalks and those pinned through the apical end. Only 10% in the latter group showed primary regeneration, contrasted with 63–73% in the former groups. It appears that the apico-basal gradient is not changed when pinning is basal or central but that the tendency of the oral end to regenerate apical structures is inhibited by pinning through that end.

C. *Fusion of pieces*

It was possible by the technique described under Methods to fuse portions of animals in various combinations, including (1) apical ends together, (2) the apical end of one against the basal end of the other, (3) basal ends together, and (4) a portion of one animal between portions of two other animals (Fig. 6). The particular combinations tried are outlined in Table II.

TABLE II

Fusion of portions of hydranths

Type of fusion	No.	Age in days after fusion	Results
A. Two pieces			
1. Apical-to-apical			
a. Hypostomes removed; tentacular regions joined	1	13	No primary regeneration. Dedifferentiation of tentacular and gonosomal regions
b. Hypostomes and tentacular regions removed; gonosomes joined	6	13	No primary regeneration. Dedifferentiation of gonosomal region
*c. Stems only	15	7	No primary regeneration
*2. Apical-to-basal (stems only)	9	7	No primary regeneration
*3. Basal-to-basal (stems only)	20	7	No primary regeneration
B. Three pieces Pieces numbered below were fused between basal ends of stalks of 2 complete hydranths			
1. Hypostome	4	10	No primary regeneration. Incorporation into stems
2. Tentacular region	5	10	Extra tentacles in tentacular region; later dedifferentiation
3. Gonosomal region	7	12	No primary regeneration. Dedifferentiation of gonosome
4. Stem	5	19	No primary regeneration, no apparent dedifferentiation

* Pinned through middle of one member.

Several generalizations emerge from an examination of the results recorded in Table II. The first is that primary regeneration of parts excised did not take place when portions of hydranths were fused in apical juxtaposition (Experiment A-1). Primary regeneration could have occurred at the apical end if these parts had been isolated separately. This result illustrates the importance of environment in determining morphogenesis at the cut surfaces of hydranths. A similar general explanation would account for the results of fusing pieces between two other hydranths (Experiments B-1, B-2, B-3 and B-4). Although the grafted piece did undergo slight regeneration in one series (extra tentacles in Experiment B-2), generally it merely underwent dedifferentiation. There was no evidence of an inductive effect of the graft on the differentiation of adjacent regions of the host stems.

A third result is that obtained in the fusions of stems in apical-to-basal or basal-to-basal orientation. In these, either one (Experiment A-2) or two (Experiment A-3) apical ends were freely exposed to the culture medium. One member of the fused pair of stems was pinned through the middle to the substratum as in the simple experiments of isolation. In none of the fused pairs, however, was there any evidence of primary regeneration of tentacles at the free apical end.

Dedifferentiation of tentacular and gonosomal regions in Experiments A-1a and A-1b, B-2 and B-3 appeared to be a response to unfavorable physical and chemical conditions, perhaps comparable to those accounting for dedifferentiation of isolated single hydranths exposed to a hypotonic medium or failing to become attached to the substratum.

Discussion

The experiments reported in this paper have raised several interesting problems. First of all, we should like to understand the differences between what we have called "primary" and "secondary" regeneration. In primary regeneration the apico-basal gradient already established apparently is maintained, and a single new oral end develops within a few days at the apical end of a cut piece. Secondary regeneration depends upon a more profound dedifferentiation of existing structure or upon the outgrowth of hydrorhiza, and therefore takes several days longer than primary regeneration. Several new hydranths may develop in close proximity in secondary regeneration, indicating that the dedifferentiated region has become equipotential. These two types of regenerative response were also encountered by Goldin and Barth (1941) in their experiments on regeneration of expressed coenosarcal fragments of Tubularia as compared with stems retaining the perisarc, and by Brien (1943) in *Clava squamata*. Further investigations, both histological and physiological, are required to reveal how the cells actually behave during both primary and secondary regeneration.

A second problem is to explain why regeneration is favored by attachment to the substratum. Normally, of course, Clava is attached basally and may elongate or contract. An isolated stem continues to exhibit these movements, but it frequently rounds up after a few hours, ceases its motility and after several days degenerates without any sign of regeneration. Possibly apical and basal ends in rounded specimens are brought so closely together that the apico-basal gradient is abolished; perhaps internal pressure inhibits differentiation of new oral structures. Attach-

ment by pinning obviously provides the stem with a practical substitute for its normal attachment, thereby enabling elongation and contraction, conditions which appear to be favorable for primary regeneration. As for secondary regeneration, attachment is a *sine qua non*. Hydrorhiza must have a substratum for continued outgrowth and dedifferentiated pieces need to be attached in order to put out new hydranths.

Two additional problems are raised by the inhibition of primary regeneration in (1) isolated stems pinned through the apical end or (2) stems fused together. In the pinning experiments it may be guessed that the glass rod mechanically changed the environment of the apical cells so that they could not move to the positions usually taken in regeneration or could not divide so readily. Another possible explanation is that the apical end pinned close to the substratum was deprived of oxygen or exposed to accumulated waste products in such concentration that regeneration was inhibited. Experiments on the effect of pinning on the respiratory metabolism of Clava might help resolve this problem. Inhibition by fusion is not unexpected in the experiments of joining two pieces with apical ends together or fusing pieces between two other stems. Cells which, if exposed to the culture medium, would form part of regenerated oral structures would in fusion be joined with neighboring cells and mechanically prevented from organizing into a regenerate. More difficult to explain, however, is the inhibition of primary regeneration in stems united in apical-to-basal or basal-to-basal orientation. The data presented in Table II are too few to give any clear idea of the mechanism of inhibition. The long (12 hours) process of fusion on a glass rod may have altered the stems in some manner inimical to regeneration. Unfortunately, the control experiment of holding a single stem on a glass rod for up to 12 hours before culturing was not tried. It is possible that under the conditions of the experiments there was an oxygen deficiency. The experiments ought to be repeated with larger numbers of animals and with particular attention to environmental conditions known to favor regeneration, namely, pH, temperature, oxygen and removal of waste products.

Summary

1. Observations have been made on regeneration of isolated pieces of hydranths of *Clava leptostyla,* including the hypostome and tentacle-bearing region, the gonosome and the stalk.

2. Stalks isolated in 50% and 80% sea water underwent accelerated dedifferentiation as compared with controls in normal sea water.

3. The distinction is drawn between (1) "primary" regeneration, meaning the differentiation of missing oral or basal ends under the influence of the existing apico-basal gradient and (2) "secondary" regeneration, the delayed development of new hydranths from attached hydrorhiza or dedifferentiated tissue.

4. Primary regeneration at the apical end of isolated stalks usually occurred after anchoring the stem against 2% agar with a fine glass rod thrust through the middle or the basal end. Regeneration was inhibited, however, if the apical end was pinned.

5. Primary regeneration of apical ends was repressed by fusion of pieces of 2 hydranths in apical-to-apical juxtaposition or by fusion of an apical piece between the basal ends of two other hydranths.

6. Apical regeneration likewise failed to occur in fusions of two stalks in apical-to-basal or basal-to-basal orientation.

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